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Expression of an environmentally friendly synthetic protein-based polymer gene in transgenic tobacco plants

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ABSTRACT

We report the expression of a protein-based polymer (Gly-Val-Gly-Val-Pro)$_{121}$, i. e., (GVGVP)$_{121}$ in transgenic tobacco (*Nicotiana tabacum* var. Kentucky 17) plants. The plant expression vector pBI121-XZ-120mer which contains the gene (GVGVP)$_{121}$ with a prokaryotic preferred codon composition driven by the CaMV 35S promoter was introduced into tobacco plants by *Agrobacterium*-mediated transformation. Stable integration of the (GVGVP)$_{121}$ polymer gene was confirmed by Southern blot analysis. Northern hybridization showed polymer transcripts in leaves of transgenic plants. The (GVGVP)$_{121}$ polymer protein was detected in leaves of transgenic plants by Western blot. The (GVGVP)$_{121}$ protein could be easily purified to a high degree of purity from leaves of transgenic plants by reversible phase transition as revealed by SDS-PAGE gels stained by $CuCl_2$. Transgenic plants grew, flowered, and produced seeds normally.

INTRODUCTION

Environmental problems require the development of biodegradable materials which can be produced from renewable resources without the use of toxic and hazardous chemicals, and which will help solve the increasing global solid waste disposal burden. Among such materials are protein-based polymers. Elastic and plastic protein-based polymers, defined as high polymers of repeating peptide sequences, offer a range of materials similar to that of petroleum-based polymers, such as hydrogels, elastomers, and plastics. Protein-based polymers have their origins in repeating sequences that occur in all sequenced mammalian elastin proteins (Yeh et al. 1987). In the most striking examples, the sequence (Val^1-Pro^2-Gly^3-Val^4-Gly^5)n occurs in bovine elastin with n=11, without a single substitution (Yeh et al. 1987).

Protein-based polymers can be prepared of varied design and composition and can be made biodegradable with chemical clocks to set their half lives (Urry 1995) so that they can be environmentally friendly. Protein-based polymers tested to date have been shown to have remarkable biocompatibility, thereby enabling a whole range of medical applications including the prevention of post-surgical adhesions, tissue reconstruction, and programmed drug delivery (Urry et al. 1993). For example, the polymer poly (GVGVP), used in this study, has been shown to prevent adhesions in the rat contaminated peritoneal model following abdominal injury (Urry et al. 1993). On the non-medical side, potential applications include transducers, molecular machines, superabsorbant agents, biodegradable plastics, and controlled release of agricultural crop enhancement agents, such as herbicides, pesticides, growth factors, and fertilizers.

Initially, protein-based polymers were prepared by chemical means using either classical solution syntheses or solid phase syntheses (Urry et al. 1985). As the longer repeats have begun to be designed with more functional moiety, however, the syntheses have become so difficult that genetic engineering and bioproduction becomes an increasingly attractive alternative even for research quantities of materials. Furthermore, the cost of chemical synthesis is too high, in addition to the use of undesirable noxious and hazardous chemicals.

Clearly, commercial viability of such protein-based polymers requires a cost of production that would begin to rival that of oil-based polymers. The potential to do so resides in low cost bioproduction. So far several protein-based polymers have been produced in *E. coli* through genetic engineering (McPherson et al. 1992; Krejchi et al. 1994; Urry et al. 1996; Guda et al. 1995; Daniell et al. 1996; McPherson et al. 1996). In our previous study, a synthetic polymer gene, coding for (GVGVP)$_{121}$, was hyper expressed in *E. coli* to the extent that polymer inclusion bodies occupied nearly 80-90% of the cell volume (Urry et al. 1996; Daniell 1996; Guda et al. 1995;

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Daniell et al. 1996). However, current production through fermentation is still an expensive process and not competitive with petroleum-based polymers. A possible strategy for reducing the production cost would be to produce polymers in plants, because plants are cheap to grow on a large scale. While several plant systems might be considered for polymer production, tobacco seems to be preferable because of its relative ease of genetic manipulation and an impending need to explore alternative uses for this agricultural commodity. Furthermore, production of protein-based polymers in tobacco plants will open the possibility of converting other surplus agricultural products into high value-added industrial products. In our previous study, we explored the possibility of producing protein-based polymers by expressing the $(GVGVP)_{121}$ polymer in cultured cells of tobacco (Zhang et al. 1995). In this study we investigate the potential for production of this protein-based polymer in transgenic tobacco plants and develop a polymer purification methodology from transgenic leaves.

MATERIALS AND METHODS

Construction of pBI121-XZ-121mer. The initial gene which encodes 10 repeating units of the elastomeric pentapeptide Gly-Val-Gly-Val-Pro, i. e., $(GVGVP)_{10}$ was constructed by using synthetic oligonucleotides. The gene was amplified by using polymerase chain reaction (McPherson et al. 1992). Higher molecular weight polymer genes were then made by concatenation/ligation reaction using suitable adaptor oligonucleotide fragments. Details of a series of these gene constructs have been published elsewhere (McPherson et al. 1996). These higher molecular weight polymer genes were subsequently cloned into pUC118 as a BamHI-BamHI fragment. The vector for tobacco transformation was constructed as follows. The *uid*A gene was removed from the plasmid pBI121 (Jefferson et al. 1987) as a XbaI-SstI fragment and replaced by the $(GVGVP)_{121}$ gene (obtained as XbaI-SstI fragment from the pUC118 plasmid) resulting in the construct pBI121-XZ-121mer (Fig. 1).

Tobacco transformation. Binary vector pBI121-XZ-121mer was transformed into *Agrobacterium tumafaciens* strain LBA4404 (kindly provided by Dr. E. W. Nester, University of Washington) by freeze-thaw method (An et al. 1988). The construct was introduced into *Nicotiana tabacum* var. Kentucky 17 using the leaf-disc transformation method (Horsh et al. 1985). Transgenic plants were selected on MS104 medium containing 100 mg/ml kanamycin and rooted on MSO medium containing 100 mg/ml kanamycin. Plants were then transferred to soil and analyzed. For details of media composition please see Daniell (1996).

Southern blot analysis. Total DNA was extracted from leaves of 2-week-old transformed and untransformed plants essentially as described by Rogers and Bendich (1988). Total DNA (10 µg) was digested with EcoRI, separated through a 0.7% agrose gel, and transferred to a MSI membrane (Micron Separation Inc. Westboro, MA). Prehybridization and hybridization were conducted according to Daniell et al. (1995). A ^{32}P-labeled 1.8 kb $(GVGVP)_{121}$ gene fragment was prepared by the random-primed labeling procedure (Promega) and used for hybridization.

Northern blot analysis. Total RNA was isolated from 3-week-old transformed and untransformed tobacco plants essentially as described by De Vries et al. (1988). RNA (20 µg) was denatured by formaldehyde treatment, separated in 1.2% agrose gel in the presence of formaldehyde, and transferred to MSI (Micron Separation Inc. Westboro, MA). The blot was prehybridized and hybridized as described above for Southern blot analysis.

Protein isolation and immunoblotting. Plant leaves were ground in liquid nitrogen and homogenized in one volume of the extraction buffer containing 50 mM Tris, pH 7.5, 1% 2-mecaptoethanol, 5 mM EDTA, and 2 mM phenylmethyl-sulfonyl fluoride (PMSF). Insoluble debris was removed by centrifugation at 10,000 g for 15 minutes (4°C). The pellet was discarded, and aliquots (100 µg) of the supernatant were loaded onto 10% SDS-PAGE gel according to Laemmli (1970). After electrophoresis, proteins were transferred to a nitrocellulose membrane electrophoretically in 25 mM Tris, 192 mM glycine, 5% methanol (pH 8.3). The filter was blocked with 2% dry milk in Tris-buffered saline for two hours at room temperature and stained with antiserum raised against the polymer AVGVP (kindly provided by the University of Alabama at Birmingham, monoclonal facility) overnight in 2% dry milk/Tris buffered saline. The protein bands reacting to the antibodies were visualized using alkaline phosphatase-linked secondary antibody and the substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Bio-Rad). Quantification of purified polymer proteins was carried out by densitometry using Scanning Analysis software (BioSoft, Ferguson, MO) installed on a Macintosh LC III computer (Apple Computer, Cupertino, USA) with a 160-Mb hard disk operating on a System 7.1, connected by SCSI interface to a Relisys RELI 2412 Scanner (Relisys, Milpitas, CA) (Wang et al. 1995). Total protein contents were determined by the dye-binding assay of Lowry et al. (1951) using reagents supplied in kit from Bio-Rad, with bovine serum albumin as a standard.

Polymer protein purification and quantitation. Plant leaves were ground in liquid nitrogen and homogenized in one volume of the extraction buffer containing 50 mM Tris-HCl, pH 7.5, 1% 2-mecaptoethanol, 5 mM EDTA, 2 mM PMSF, and 0.8 M NaCl. The homogenate was then centrifuged at 10,000 g for 10 minutes (4°C), and the pellet was discarded. The supernatant was incubated at 42°C for 30 minutes and then centrifuged immediately for 3 minutes at 5,000 g (room temperature). The pellet was resuspended in the extraction

buffer and incubated on ice for 10 minutes. The mixture was centrifuged at 12,000 g for 10 minutes (4°C). The supernatant was collected and stored at -20°C. The purified polymer protein was electrophoresed in a SDS-PAGE gel according to Laemmli (1970) and visualized by either staining with 0.3 M CuCl2 (Lee et al. 1987) or transferred to nitrocellulose membrane and probed with antiserum raised against the polymer protein as described above for Western blot analysis. Quantification of purified polymer proteins was carried out by densitometry as described above.

RESULTS

Vector construction and tobacco plant transformation. An expression vector, pBI121-XZ-120mer (Fig. 1), containing the $(GVGVP)_{121}$ coding sequence driven by the cauliflower mosaic virus (CaMV) 35S promoter and flanked by the nopline synthase (*nos*) gene terminator was made. This vector also contains the *npt*II gene driven by the *nos* promoter and flanked by the *nos* terminator to facilitate selection on kanamycin. This plasmid was introduced into tobacco plants by *Agrobacterium*-mediated transformation. Thirty five independent tobacco plants were regenerated. These plants were screened for the presence of the $(GVGVP)_{121}$ polymer gene by Southern blot analysis. Southern analysis confirmed that the $(GVGVP)_{121}$ gene was stably integrated into the genome of more than 50% of regenerated tobacco plants tested and the number of gene copies was estimated to be one to three (data not shown). However, majority of the transgenic plants contained only one copy of the integrated polymer gene.

Analysis of gene expression in transgenic tobacco leaves. Northern blot analysis was performed to determine if the $(GVGVP)_{121}$ polymer gene was transcribed in transgenic tobacco plants identified by Southern blot analysis. RNA was isolated from 3-week-old leaves of untransformed and transformed plants. Nine out of eighteen transgenic plants tested showed a hybridizing transcript of the expected size (1.8 kb) and the control untransformed did not show this band (Fig. 2). Four transgenic plants (# 10, #11, #14, and #15) were selected for further analysis. The $(GVGVP)_{121}$ polymer protein was detected by Western blot analysis with antibody raised against the polymer (AVGVP)n, which cross-reacts fairly strongly with (GVGVP)n. Expression levels were calculated from the Western blot by comparison to known amount of the $(GVGVP)_{121}$ polymer purified from *E. coli* bacterial cultures. The average protein levels were estimated to be 0.01% to 0.05% of total soluble leaf proteins (data not shown). However, these amounts should be regarded as an underestimation, since there are no ideal colorimetric assays for determination of the concentration of the $(GVGVP)_{121}$ polymer protein purified from *E. coli*, which was used as the standard. The polymer protein could not be assayed by the Bradford dye-binding procedure due to its lack of basic and aromatic amino acid residues (Bradford 1976). As with Lowry assay, color development is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine, and histidine (Lowry et al. 1951; Peterson 1979), which are absent in the $(GVGVP)_{121}$ polymer protein. Therefore, accurate quantitation could not be obtained by the Bradford and Lowry methods using bovine serum albumin as a standard.

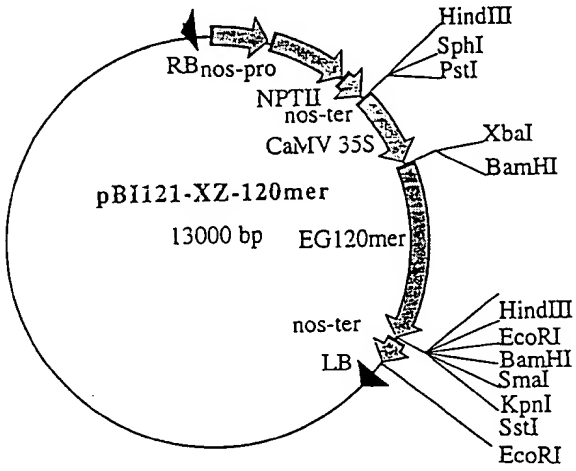

Figure 1. The plasmid pBI121-XZ-120mer. Abbreviations used in the plasmid map are: RB, T-DNA right border; nos-pro, nopline synthase gene promoter; NPTII, neomycin phosphotransferase gene that serves as a selectable marker; nos-ter; nopline synthase gene terminator; CaMV 35S, cauliflower mosaic virus 35S promoter; EG120mer, coding sequence for $(GVGVP)_{121}$; LB, T-DNA left border.

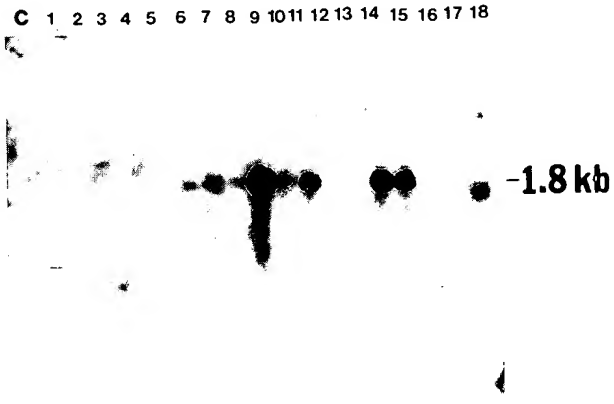

Figure 2. Northern blot analysis of tobacco leaves from contro (C) and eighteen transgenic plants (1-18) using th $(GVGVP)_{121}$ gene. A transcript (1.8 kb) is evident i transgenic plants #6, #7, #8, #9, #10, #11, #14, #15, #18.

Purification of $(GVGVP)_{121}$ from transgenic tobacco leaves by temperature induced aggregation. The Polymer $(GVGVP)_{121}$ protein exhibits the property of being soluble in water at temperatures below 25°C, but aggregates into more-ordered, viscoelastic state, called a coacevate, at 37°C. This process of increasing order on increasing temperature is called an inverse temperature transition (Urry et al. 1993; McPherson et al. 1992; McPherson et al. 1996). Increasing salt concentration such as NaCl, NaBr, Na_2CO_3, and Na_3PO_4 (in order of potency) can lower the transition temperature (Urry et al. 1993). Based on this interesting and unique property, the $(GVGVP)_{121}$ and $(GVGVP)_{251}$ polymers have been purified from *E. coli* in earlier studies (Urry et al. 1994; Guda et al. 1995; Daniell et al. 1996); we report here a protocol to purify the $(GVGVP)_{121}$ polymer protein from transgenic tobacco leaves. Polymer protein purified from one gram and five grams of mature leaf is shown in Fig. 3A and Fig. 4, respectively. By introducing a single step of high temperature (42°C) treatment and including 0.8 M NaCl in the extraction buffer, high degree of purity of $(GVGVP)_{121}$ polymer protein was obtained as revealed by SDS-PAGE gels. As discussed above, polymer protein could not be visualized by Coomassie staining due to its lack of aromatic side chains and negative staining by $CuCl_2$ was used to visualize polymer protein $(GVGVP)_{121}$. The purified $(GVGVP)_{121}$ was further confirmed by Western blot analysis (Fig. 3B). The yield of the polymer protein $(GVGVP)_{121}$ was determined by comparison to a known amount of $(GVGVP)_{121}$ purified from *E. coli.* An estimated quantity of polymer protein between 0.5 µg and 5 µg could be extracted form one gram of fresh weight of leaf tissues (approximately 0.003% to 0.03% of total soluble proteins). Clearly, only a portion of polymer proteins was extractable, as compared with the quantity (0.01-0.05 % of total soluble protein) detected by the Western blot. Therefore, effort needs to be made to maximize recovery of $(GVGVP)_{121}$ from leaves by temperature-induced polymer aggregation. Demonstration of purification by inverse temperature transition attests to the successful synthesis of the $(GVGVP)_{121}$ polymer protein in tobacco plants.

Polymer $(GVGVP)_{121}$ protein was also purified from senescing leaves. The yields from old leaves were comparable to those from growing mature leaves (data not shown). This indicated that polymer protein is stable and not degraded by proteases present in plants. Apparently, there are no common protease cleavage sites in the poly GVGVP amino acid sequence.

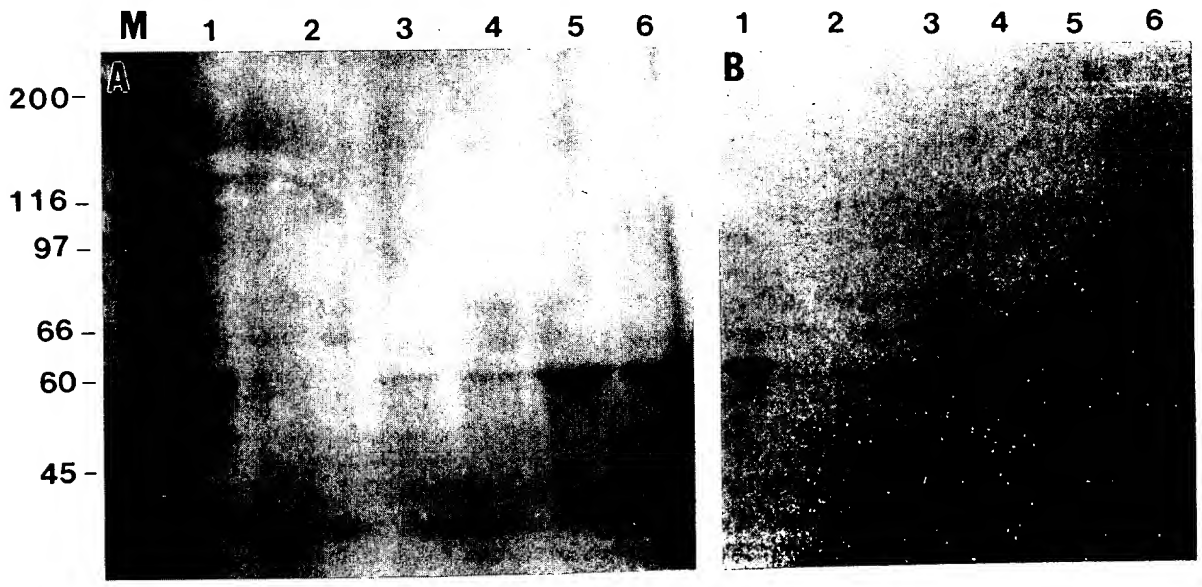

Figure 3. **A**. Copper-stained SDS-PAGE of the $(GVGVP)_{121}$ polymer protein purified from one gram (fresh weight) of leaves of plants transformed with the $(GVGVP)_{121}$ polymer gene. (M) molecular weight markers (KDa); (1) approximately 5 µg of the $(GVGVP)_{121}$ polymer protein purified from *E. coli*. (2) untransformed tobacco. (3) transgenic tobacco #10. (4) transgenic tobacco #11. (5) transgenic tobacco #14. (6) transgenic tobacco #15. **B**. Immunoblot of the SDS-PAGE identical to A. The blot was probed with an antibody raised against poly (AVGVP).

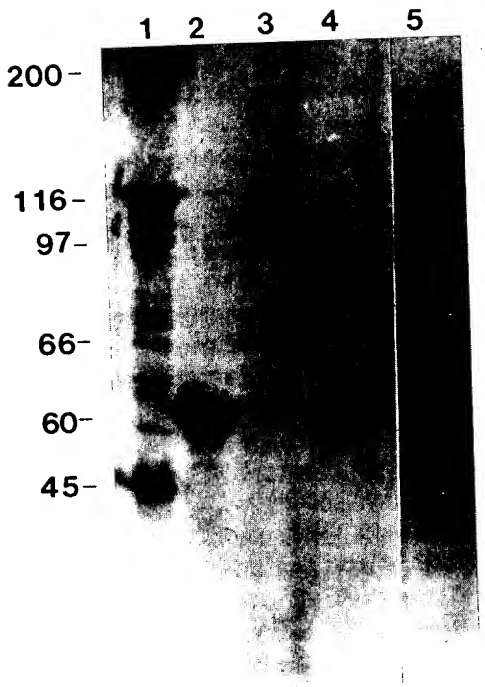

Figure 4. Copper-stained SDS-PAGE of the $(GVGVP)_{121}$ polymer protein purified from five gram (fresh weight) of leaves of plants transformed with the $(GVGVP)_{121}$ polymer gene. (1) molecular weight markers (kDa); (2) approximately 60 µg of the $(GVGVP)_{121}$ polymer protein purified from *E. coli;* (3) untransformed tobacco; (4) transgenic tobacco #11; (5) transgenic tobacco #15.

Polymer $(GVGVP)_{121}$ expressing transgenic tobacco plants did not show any phenotypic differences from untransformed plants. Transgenic plants grew, flowered and produced seeds normally. It appears, therefore, that expression of the protein-based polymer protein does not affect the growth and development of transgenic plants. Studies are in progress to further evaluate the transgenic plants and their progeny.

DISCUSSION

Plants have been used as factories for the production of biopolymers. The bacterial polyhydroxybutyrate pathway, containing three genes coding for a biodegradable thermoplastic polymer (PHB) from *Alcaligenes eutrophus*, has been successfully engineered into *Arabidopsis* plants (Nawrath et al. 1994; Poirier et al. 1992). In another study (Ebskamp et al. 1994), tobacco plants have been genetically engineered to produce fructose polymers, which have potential application in food and non-food products, by introducing a bacterial fructosyltransferase gene from *Bacillus subtilis.* However, the exact nature of the polymer produced in transgenic plants through metabolic engineering is unknown, such as the presence of monomers, molecular weight, and branching pattern of polymers, which may influence the physical properties of the end product. Unlike metabolic engineering to express other types of polymers, expression of protein-based polymer genes in this study requires the use of only a single synthetic gene and polymer proteins are direct gene products.

Another important feature of the $(GVGVP)_{121}$ polymer is its inverse temperature transition property. This makes it easier and cheaper to harvest polymers in aqueous solutions simply by raising temperature, and therefore avoid the cumbersome purification procedure and the use of enzymes and organic solvents which may alter the quality of the polymer. Extraction of $(GVGVP)_{121}$ from plants is also an important factor affecting the production cost. The ease of isolation certainly will increase the potential of $(GVGVP)_{121}$ production in plants. Yet another feature of the $(GVGVP)_{121}$ polymer is its stability, presumably because of the lack of common protease specific cleavage sites in the (GVGVP) amino acid sequence.

This study represents only the first step to produce a protein-based synthetic polymer gene product in plants. A major issue is the low level of production. High expression must be achieved in order to produce protein-based polymers on an agricultural scale. There are several reasons for the low level accumulation of the polymer protein. One possible explanation could lie in the polymer coding sequence, which has a prokaryote-preferred codon composition. Previous studies have shown that modifications of the DNA sequence of prokaryotic genes can significantly enhance expression of bacterial transgenes in higher plants (Wunn et al. 1996; Koziel et al. 1993; Perlak et al. 1991). For example, a major hurdle in engineering insect resistant plants has been the low level of expression of the *Bacillus thuringiensis* (B. t.) toxin gene; increase in B. t. toxin gene expression of up to 500-fold has been achieved through specific modification of the B. t. coding sequence to suit the eukaryotic nature of plant nuclei (Perlak et al. 1991). Therefore, a parallel expression study using the poly (GVGVP) gene with the tobacco nuclear-preferred codons is currently being conducted in our laboratory. Additionally, availability of the amino acid pool could also be a limiting factor for high level expression of this polymer gene. Earlier experiments have demonstrated that amino acid pools exist in the chloroplast (Goodwin 1971). Especially, the enzymes involved in formation of valine (Schulze-Siebert et al. 1984) and proline (Rayapati et al. 1989) are located in the chloroplast. Therefore, another possible strategy for

overcoming this problem is to change the subcellular location of $(GVGVP)_{121}$ synthesis to chloroplasts. The previous study indeed showed that the maximal level of polyhydroxybutyrate (PHB) production was increased 100-fold by changing the location of PHB production from a cellular compartment with a low flux of acetyl-CoA (cytoplasm) to a compartment with high flux of acetyl-CoA (plastid) (Nawrath et al. 1994), and the low PHB yield by cytoplasmic expression of the PHB biosynthetic enzymes could be explained by the limited supply of cytoplasmic acetyl-CoA available for PHB synthesis.

The present study demonstrated the feasibility of expressing protein-based polymers in plant systems, however, production of a large quantity of $(GVGVP)_{121}$ will require additional genetic manipulation, such as optimization of codon usage or expression of the $(GVGVP)_{121}$ polymer gene in the chloroplast compartment.

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